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13. ABSTRACT (Maximum 200 Words)

Long-term goal of this application was to elucidate pathogenetic mechanisms in human autoimmune paraneoplastic neurological conditions. These syndromes result from the ectopic expression in cancer cells of neuronal antigens followed by the onset of an autoimmune response directed at the cancer tissue. A side effect of this response is an autoimmune impairment of nervous system function. We have identified novel autoantigens of autoimmune paraneoplastic conditions, primarily autoantigens expressed in breast cancer. Recently, we have identified a woman with lower motor neuron syndrome, breast cancer and anti-βIV spectrin autoantibodies. The specific goal of this project was to conduct studies on the properties of βIV spectrin and to determine whether anti-βIV spectrin antibodies are a frequent occurrence in cancer. If this was the case, such antibodies could be used as diagnostic tools and βIV spectrin could represent a target for cancer immunotherapy. We have made progress towards the elucidation of βIV spectrin function and expression profile. A βIV mutant mouse was generated and found to have significant neurological defects, consistent with a critical role of this protein in axonal physiology. However, so far we have failed to obtain evidence for the occurrence of widespread anti-βIV spectrin autoimmunity in cancer patients.

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Introduction

Long-term goal of the project was the elucidation of autoimmune immune mechanisms in cancer with the expectation that such studies will make possible the development of diagnostic tools for the early detection of cancer as well of immuno-therapeutic strategies for its cure. The rationale of the application was to capitalize on the occurrence of autoimmune paraneoplastic neurological conditions to improve our understanding of autoimmunity in cancer. These are human syndromes in which the ectopic expression in cancer of neuronal antigens, or of neuronal splice variants of ubiquitously expressed proteins, leads to autoimmune attack of cancer tissue that often delays its progression. The same attack, however, also results in an autoimmune impairment of the nervous system function. An attractive possibility is that the ectopic autoantigen may not only trigger autoimmunity but also play a role in the biology of cancer cells. Our lab has identified novel paraneoplastic autoantigens and paraneoplastic syndromes. For example, we have defined a syndrome characterized by breast cancer, Stiff-Man syndrome and autoantibodies directed against the neuronal protein amphiphysin. In some of these patients our detection of anti-amphiphysin antibodies has prompted the search for, and detection of, an occult breast cancer. Recently, we have identified a patient with breast cancer, Lower Motor Neuron Disease and autoimmunity to βIV spectrin. Upon removal of the cancer there was a 70% reduction in the titer of anti-βIV spectrin autoantibodies and a dramatic improvement of the neurological symptoms, confirming that the cancer tissue was driving the autoimmune response. βIV spectrin a member of the spectrin family of plasma membrane associated proteins, which is expressed as alternatively spliced isoforms in a variety of tissues including brain. A major brain isoform of βIV spectrin is present at axon initial segments and nodes of Ranvier where it may participate in the localization and concentrations of Na^+ channels. This project had two specific aims: 1) the characterization of the expression of βIV spectrin in normal and neoplastic tissue and 2) the screen of a large population of breast cancer patients for the presence of anti-βIV spectrin antibodies.

Body

We have pursued studies on the cell biology of βIV spectrin and on the potential connection of this protein to cancer.

We have generated a mouse that selectively lacks the longer βIV spectrin transcript (the 250 kD isoform corresponding to βIVΣ1 spectrin). All other βIV spectrin isoforms, which are smaller in size, were still detectable by immunoblot. These data confirm our hypothesis that such polypeptides do not originate from the proteolysis of βIVΣ1 spectrin, but represent alternatively spliced variants of the gene (Berghs et al., 2002). βIVΣ1 spectrin -/- mice are moderately hyperactive, and display intention tremor and mild dysmetria, consistent with cerebellar impairment and possibly some degree of abnormal myelination. The lack of βIVΣ1 spectrin correlates with a reduced immunoreactivity for Na^+ channels at nodes of Ranvier and with a broader distribution of K^+ channels in the adjacent paranodal regions (Lacas-Gervais et al., abstract for the ELSO Meeting 2003; manuscript in prepartion). These changes may explain the deficit in nerve conduction that has also been observed in two other independently described βIV spectrin -/- mice (Parkinson et al., 2001; Komada and Soriano, 2002), both of which lack multiple βIV spectrin isoforms. Our data, however, are the first to prove that the βIVΣ1 spectrin is the specific spectrin isoform expressed at nodes of Ranvier and that the absence of this isoform is sufficient to induce neurological alterations previously observed in

βIV spectrin -/- mice. Taken together,these findings are consistent with a potential role of anti-βIV spectrin autoimmunity in the generation of paraneoplastic neurological symptoms in the patient we have investigated.

βIVΣV spectrin is a 70 kD spliced variant of βIV spectrin that has been previously found in the nuclear bodies enriched in the acute promyelocytic leukemia (PML) protein, henceforth termed PML bodies (Tse et al., 2001). It has been suggested that PML bodies participate in the regulation of gene expression and/or protein degradation in the nucleus. We have now shown that other βIV spectrins are present in the nucleus and near the nuclear envelope. The small ubiquitin-like modifier SUMO and the SUMO ligases of the PIAS family are enriched in the PML bodies and several components of the PML bodies undergo sumoylation. Sumoylation has been also shown to modulate the nuclear import of cytosolic proteins. Interestingly, we identified βIV spectrin as an interactor of the receptor tyrosine phosphatase like-protein ICA512 and we have shown that the cytoplasmic domain of ICA512 binds the SUMO ligase PIASγ. A possible role of βIV spectrin in gene regulation is consistent with a potential function of this protein in the biology of cancer cells.

Concerning the second specific aim, i.e. the screen of a large population of cancer patients for the occurrence of anti-βIV spectrin autoantibodies, we lowered its priority because of the lack of sufficiently strongly preliminary results suggesting the presence of anti-βIV spectrin autoantibodies in the cancer population at large. Eventually, we did not have time to pursue it.

Support of this grant has indirectly supported additional studies on paraneoplastic autoimmunity. A case study on a patient with breast cancer, Stiff-Man syndrome, autoimmunity directed against amphiphysin and rabdomyolisis (an amphiphysin isoform is highly expressed in skeletal muscle) is appended to this report.

Key research accomplishments

- Generation and characterization of βIVΣ1 spectrin -/- mice
- Demonstration of βIVΣ1 as the specific spectrin isoform expressed at nodes of Ranvier
- Identification of a novel paraneoplastic condition associated with breast cancer

Reportable outcomes

Manuscripts

Lacas-Gervais S*, Guo J*, Knoch K-P, Rasband M, De Camilli P and Solimena M. 2003. Deletion of βIVΣ1 spectrin alters the organization of nodes of Ranvier and is sufficient to produce the "quivering" phenotype in mice (manuscript in preparation). * equal contributors.

Petzold GC, Marcucci M, Butler MH, van Landeghem, FKH, Scholz P, Einhäupl KM, Solimena M, Valdueza JM and De Camilli P. 2003. Recurrent rhabdomyolisis in a patient with Stiff-Person (Stiff-Man) syndrome associated with breast cancer and autoimmunity to amphiphysin (The manuscript, see appendix, will be submitted within the next 2-3 weeks)

Abstracts

Lacas-Gervais S*, Guo J*, Knoch K-P, Rasband M, De Camilli P and Solimena M. 2003. Quivering

phenotype and altered distribution of K^+-channels in βIVΣ1 spectrin KO mice. Abstract to the ELSO Meeting, September 2003, France * equal contributors (see appendix).

Yang Y, Lacas-Gervais S, Morest K, Solimena M, Rasband MN. Deletion of the specific and PH of βIV spectrin results in disrupted nodes of Ranvier. Abstract to the Society for Neuroscience Meeting, November 2003 (see appendix).

Developement of cell lines, tissue etc.
Generation of βIVΣ1 spectrin -/- mice
Development of antibodies specific against βIV spectrin isoforms (anti-βIVΣ1/βIVΣ2, anti-βIVΣ3, anti-βIVΣ4)

Funding applied for partially based on support by this award: we have successfully applied for a competitive renewal of an NCI Program project grant (project #1 P01 CA46128) focused on the study of the actin-based cytoskeleton in the biology of cancer cells. This grant cover the period 1-1-03/12-31-08.

Conclusions

We have conclusively shown that βIVΣ1 spectrin, i.e. the main target of autoimmunity in the patient with breast cancer and paraneoplastic lower motor neuron syndrome, is a main component of nodes of Ranvier. The properties of βIV spectrin are consistent with its pathogenetic link to the neurological symptoms.

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Appendices

Appendix 1	Manuscript (Petzold et al.)
Appendix 2	Abstract Lacas-Gervais
Appendix 3	Abstract Yang
Appendix 4	Curriculum vitae of Dr. Pietro De Camilli (PI)
Appendix 5	Curriculum vitae of Dr. Michele Solimena (PI of the original application; the grant was transferred to Dr. De Camilli when Dr. Solimena left Yale)

Appendix 1

THIS MANUSCRIPT WILL BE SUBMITTED WITHIN THE NEXT COUPLE OF WEEKS (5/27/03)

RECURRENT RHABDOMYOLYSIS IN A PATIENT WITH STIFF-PERSON (STIFF-MAN) SYNDROME ASSOCIATED WITH BREAST CANCER AND AUTOIMMUNITY TO AMPHIPHYSIN.

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Stiff-Person syndrome (Stiff-Man syndrome) is a central nervous disorder characterized by progressive muscular rigidity, superimposed episodic spasms and continuous motor unit activity at rest[2,16]. The majority of patients harbor autoantibodies against glutamic acid decarboxylase (GAD), the rate-limiting enzyme for the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid, in serum and cerebrospinal fluid[23,24]. These and other clinical, pharmacological and electrophysiological findings[14] have led to the hypothesize an autoimmune-mediated impairment of central nervous inhibitory circuits, resulting in tonic firing of α-motoneurons, as the cause for the disease. Autoreactivity directed against cells expressing GAD can also cause pathology outside the central nervous system, primarily insulin-dependent diabetes mellitus[1,15,24].

A paraneoplastic etiology, i.e. a disturbance of organ function occurring at a site remote from an underlying neoplasm or its metastases[6], is thought to underlie about 5% of patients with Stiff-Person syndrome. Even in this case, there is evidence for an autoimmune origin of the neurological symptoms, although autoimmune mechanisms may be different. These patients usually have breast adenocarcinoma [7,10,11] and are positive for autoantibodies against amphiphysin [7,10,11], a synaptically enriched protein with a putative role in membrane traffic, signalling and actin function[9]. Amphiphysin 1 is expressed only at very low levels outside the brain, but can be overexpressed in breast cancer[10]. In brain, amphiphysin 1 exists primarily as a heterodimer with amphiphysin 2 (neuronal amphiphysin 2 or n-amphiphysin 2), a similar protein encoded by a different gene [9,18,27]. An amphiphysin 2 splice variant (muscle amphiphysin 2 or m-amphiphysin 2, also referred to as Bin1[21,26] is expressed at very high level in skeletal muscle, where it is thought to function in transverse-tubule physiology[3,12,19].

Here we report a patient (Pt #1) with breast cancer, paraneoplastic Stiff-Person syndrome and recurrent rhabdomyolysis. We demonstrate that the serum of the patient contains antibodies directed not only against amphiphysin 1, but also amphiphysin 2, suggesting a pathogenetic link between rhabdomyolysis and anti-amphiphysin 2 autoimmunity.

Case report

A 62-year-old woman was admitted for acute fever, malaise, severe generalized myalgia and dark-coloured urine. Initial laboratory tests revealed hyperkalemia, myoglobinuria and highly elevated serum levels of creatine kinase and myoglobin. She had not taken any medication or recreational drugs. Recent history was negative for infectious diseases or strenuous exercise. Family history was unremarkable. These clinical and laboratory findings led to the diagnosis of rhabdomyolysis of unknown cause. The patient recovered after administration of intravenous fluid and high-dose methylprednisolone over several days.

Recent medical history revealed that three weeks before admission, the patient noticed a progressive rigidity and stiffness of the right proximal leg and lower back that spread to the right calf and foot and to the left leg within two weeks, resulting in bilateral fixed plantar flexion and inversion of feet and toes. She also had episodes of painful spasms in both legs aggravated by sudden noise or emotional upset that disappeared during sleep. Deep tendon reflexes were brisk and symmetrical. Plantar reflexes were flexor. The remaining neurological examination was also unremarkable. Magnetic resonance imaging of the brain and spinal cord was normal. Cerebrospinal fluid examination revealed lymphocytic pleocytosis (82 cells cm^{-3}), an increased IgG index (3.6), oligoclonal bands confined to the cerebrospinal fluid and a protein content of 0.6 g l^{-1}. Peripheral and central nervous sensory and motor conduction was within normal limits. Concentric needle electromyography of paraspinal and lower limb muscles showed continuous motor unit activity at rest. Antibodies against GAD were not detected, but serum and cerebrospinal fluid contained high titers of autoantibodies against amphiphysin 1. No other

paraneoplastic antibodies, such as antibodies directed against Yo, Hu, Ri, Ma, were detected. Based on the presence of anti-amphiphysin autoimmunity, a search for an underlying malignancy was initiated and breast cancer was suspected by mammography. Following surgical excision, tumour histopathology revealed poorly differentiated invasive ductal adenocarcinoma with regional lymph node metastasis. Further diagnostic work-up disclosed no evidence for distant metastases. Based on these findings, a diagnosis of paraneoplastic Stiff-Person syndrome was made. The episodes of painful spasms were alleviated shortly after oral treatment with clonazepam and prednisolone. However, six severe episodes of rhabdomyolysis occurred intermittently that were responsive to high-dose intravenous methylprednisolone.

The patient remained wheelchair-bound due to the plantar flexion and rigidity of both legs even after two months of oral medication. Therefore, she was treated with 400 mg kg^{-1} intravenous human immunoglobulin per day for five consecutive days, according to the treatment regimen proposed by Dalakas et al.[5]. This resulted in a profound improvement of her rigidity, allowing her to walk again with assistance. Furthermore, rhabdomyolysis ceased to occur. After the patient was discharged for rehabilitation, she continued to improve until she unexpectedly deteriorated with signs of an acute systemic infection. She was admitted to a municipal hospital and died two days later in acute septic shock.

The co-occurrence of rhabdomyolysis and paraneoplastic Stiff-Person syndrome and the remission of both after intravenous immunoglobulin therapy raised the suspicion that autoreactivity against skeletal muscle could be involved. More specifically, we considered the potential presence of autoimmunity directed against amphiphysin 2, an abundant skeletal muscle protein. Therefore, further tests were conducted.

Methods

Serum samples were obtained before intravenous immunoglobulin therapy was initiated. Specimen of brain, lumbal spinal cord and skeletal muscle were obtained at autopsy. Hematoxylin and eosin (H&E) staining, luxol fast blue-Periodic Acid Schiff (PAS) reaction and Diaminobenzidine (DAB) immunohistochemistry with antibodies against CD3, CD8, CD20, CD68 and GFAP (DAKO, Germany) were performed according to standard protocols. Western blotting of rat tissues, cell transfection, and immunofluorescene of transfected cells were performed as described[3,11,12]. The following rabbit sera were from our lab: CD8, raised against the COOH terminal region of amphiphysin 2 and specific for amphiphysin 2^3, EVA, raised against exon 7 of amphiphysin 2^{26}, and CD9, raised against a short peptide highly conserved in amphiphysin 1 and 2 and equally reactive with amphiphysin 1 and 2^3. Monoclonal antibodies specific for amphiphysin 2^{21} and for the HA epitope (3F10) were from UBI and Roche, respectively. The serum of another patient (Pt #2) with amphiphysin autoimmunity and paraneoplastic Stiff-Person syndrome was from out caseload. For the expression of human amphiphysin isoforms in CHO cells the following cDNA clones were used: Amph1-pCDNA3(HA) (amphiphysin 1), nAmph2-pCDNA3 (n-amphiphysin 2), and mAmph2-pCDNA3 (m-amphiphysin 2).

Results

Examination of central nervous tissue and skeletal muscle

At pathological examination the number of motoneurons in the anterior horn of the lumbal spinal cord was moderately reduced. Some of the motoneurons appeared shrunken and eosinophilic with varying degrees of chromatolysis (Figure 1, A). Scattered CD3- and CD8-

positive T-lymphocytes were present in proximity of neuronal cell bodies and axons (Figure 1, B, C). Neither intraneuronal inclusions nor CD20-positive B-lymphocytes nor perivascular lymphocyte cuffing could be observed. The number of CD68-positive microglial cells and macrophages was markedly increased not only within the grey matter but also in the white matter, whereas astrocytic gliosis appeared mild. In a skeletal muscle biopsy, many muscle fibers appeared severely atrophic (Figure 1, D) or were infiltrated by by CD68-positive macrophages, indicating rhabdomyolysis (Figure 1, E).

Detection of autoantibodies

When the serum of the patient was tested against a postnuclear supernatant (PNS) of brain tissue in a western blot assay, the most prominent reactivity was observed with a set of immunoreactive bands in the 120 kDa range (Figure 2, A). An additional set of bands in the 80-90 kDa region were less intensely reactive. Both the upper and lower group of bands comigrated with material recognized by rabbit sera raised against amphiphysin 1 and/or 2 (Figure 2, A). Sera CD8 and EVA are directed against amphiphysin 2, while serum CD9 recognizes equally both proteins (Figure 2, A). These results are consistent with the presence in the serum of Pt #1 of antibodies directed against both amphiphysin 1 and 2. The serum of a previously studied patient (Pt#2), with Stiff-Man syndrome, breast cancer and anti-amphiphysin autoimmunity, produced a very similar western blot pattern (Figure 2, A). Surprisingly, neither Pt #1 serum nor Pt #2 serum produced a detectable signal on amphiphysin 2 when tested by western blotting on skeletal muscle tissue (not shown), in spite of the high expression level in this tissue of m-amphiphysin 2. To clarify this discrepancy, we expressed n- and m- human amphiphysin 2 in CHO cells and tested the two patient sera by western blotting and immunofluorescence on these cells. N- and m-amphiphysin 2 have different molecular weight due to the absence in m-amphiphysin 2 of a neuron-specific insert[3,21,26]. Bin1, a monoclonal antibody raised against amphiphysin 2, demonstrated the expression of appropriate bands at approximately 85 and 60 kDa respectively, while the serum of Pt #1 (and that of Pt #2 used as a control) reacted only with n-amphiphysin 2 (Figure 2, B). In contrast, Pt #1 serum produced a strong fluorescence pattern not only on n-amphiphysin 2 (and amphiphysin 1) expressing cells, but also m-amphiphysin 2 expressing cells (Figure 3). We conclude that Pt #1 serum recognizes only conformational epitopes in m-amphiphysin 2, which are disrupted by the denaturing steps involved in western blotting, but not by the formaldehyde fixation used for immunofluorescence. In agreement with this conclusion, Pt #1 serum only reacted with a pool of m-amphiphysin 2 in transfected cells. It reacted with the diffuse cytosolic pool of the protein but not the linear elements representing amphiphysin 2 coats around tubular invaginations of the plasma membrane induced by its overexpression (Figure 3, insets). The reactivity of Pt #1 serum with n-amphiphysin 2 in western blot assays must reflect the reactivity contributed by the neuron-specific exons of the protein.

Discussion

We describe here a patient affected by breast cancer, paraneoplastic Stiff-Person syndrome and rhabdomyolysis. The presence of anti-amphiphysin autoimmunity is consistent with an autoimmune origin of the neurological symptoms. In addition, the response of rhabdomyolysis to steroid treatment and immunoglobulin therapy, together with histological examinations, suggest an autoimmune origin of this condition as well. Moreover, we show here that our patient was positive for antibodies that recognize native epitopes of m-amphiphysin 2, a very abundant protein of skeletal muscle, in addition to antibodies reactive with native and denatured epitopes of neuronal amphiphysin isoforms. These finding raise the possibility that an autoimmune process triggered by cancer may have attacked both brain and muscle, thus defining

a new paraneoplastic autoimmune syndrome: breast cancer associated with Stiff-Person syndrome, rhabdomyolysis and anti-amphiphysin autoimmunity. The breast cancer tissue of the patient studied here was not available for biochemical testing. However, the expression of amphiphysin in some cases of breast cancer was documented previously[10], consistent with a role of this protein in the triggering of autoimmunity.

In earlier reports of paraneoplastic neurological cases associated with anti-amphiphysin autoimmunity, primarily Stiff-Person syndrome or similar conditions, autoantibodies were found to be directed exclusively or primarily against amphiphysin 1[7,10,11]. However, typically, antibodies were searched for by the western blot assays, a method which involves protein antigen denaturation and therefore disrupts reactivity of conformation-sensitive epitopes. Even in the case described here, antibodies revealed by western blotting revealed primarily anti-amphiphysin 1 antibodies. Antibodies directed against amphipysin 2 were of lower titre and, in the case of m-amphiphysin 2, recognized only native epitopes. Yet, this observation is not inconsistent with a close and direct relationship between anti-amphiphysin autoimmunity and rhabdomyolysis in our patient. A similar situation exists in the case of the association between autoimmunity directed against glutamic acid decarboxylase (GAD), Stiff-Person syndrome and insulin-dependent diabetes mellitus (IDDM). Autoimmunity directed against GAD is the known link between Stiff-Person syndrome and IDDM. However, in most cases of IDDM only anti-GAD antibodies directed against native epitopes are present, while GAD autoantibodies of Stiff-Man syndrome typically react very strongly in western blot assays[1,15].

Both amphiphysin 1 and 2 are intracellular antigens, and the potential pathogenetic role of anti-amphiphysin autoantibodies remains elusive. A wide variety of other human autoantibodies associated with diseases, including autoantibodies of other paraneoplastic conditions[6], GAD autoantibodies[1,23] and antibodies found in a variety of organ-specific and systemic autoimmune diseases[4] are also directed against intercellular antigens. Cleary, there is a relationship between the autoantibodies and the specific pathological condition, but the relation may be only indirect. This also explains why the induction of humoral immunity directed against these antigens is not sufficient to elicit disease (for example in experimentally immunized animals) and why not all patients with autoantibodies develop disease or the whole spectrum of diseases that can be associated with a given autoantibody.

Whereas the ability of autoantibodies associated with infectious diseases to precipitate rhabdomyolsysis is well known[28], so far no autoantibodies have been identified in cases of rhabdomyolysis associated with neoplasias[8,13,17,20,22,25]. Anti-amphiphysin 2 autoimmunity may be present in some of these cases. Our study identifies an important, hitherto unrecognized complication in paraneoplastic Stiff-Person syndrome. When associated with Stiff-Person syndrome, rhabdomyolysis may have an autoimmune origin and a paraneoplastic etiology should be considered. It is also possible that paraneoplastic rhabdomyolysis may occur in the absence of Stiff-Person syndrome. Expeditious management is critical in rhabdomyolysis, a potentially life-threatening event[28]. Our findings suggest that immunosuppressive or immunomodulatory therapy should be considered in cases associated with Stiff-Person syndrome or with cancer.

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Figure legends

Figure 1. Post-mortem histology of the anterior horn of lumbal spinal cord (A-C) and skeletal muscle (D, E). In the anterior horn of the spinal cord, many neurons with signs of eosinophilic degeneration (A, arrow) were observed. Some CD3-positive (B) or CD8-positive (C) T-lymphocytes (arrows) were located in perineuronal or periaxonal positions. Arrowheads indicate neuronal cell bodies. Muscle biopsy shows atrophic muscle fibers (D, arrows) and myophagocytosis (E). Necrotic muscle fibers contain CD68-positive macrophages (E, arrows). H&E x400, DAB immunohistochemistry (brown) (x500).

Figure 2. Western blot analysis of Patient 1 (Pt #1) serum demonstrating presence of anti-amphiphysin antibodies. (A) A rat brain post-nuclear supernatant (PNS) was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with human sera or antibodies directed against amphiphysin. CD8 abd EVA are rabbit polyclonal antibodies raised against amphiphysin 2, and CD9 is a rabbit polyclonal antibody that equally recognizes amphiphysin 1 and 2. Pt #1 serum, like Pt#2 serum, recognize a set of bands which precisely comigrate with amphiphysin 1 and 2 immunoreactivities recognized by the rabbit antibodies. Control = secondary antibody alone. (B) Western blots of CHO cells transfected with neuronal (nAmph2) and muscle (mAmph2) amphiphysin 2 with the sera of Pt #1 and Pt #2 or with the mouse monoclonal antibody Bin1, which recognizes both amphiphysin 2 isoforms. The two human sera recognize n-amphiphysin 2 but not m-amphiphysin 2 in this assay that involves protein denaturation. Naive = untransfected cells.

Figure 3. Patient 1 (Pt #1) serum recognizes native muscle amphiphysin 2. CHO cells were transfected with neuronal or muscle isoforms of human amphiphysin 1 and 2 and then stained by immunofluorescence with the serum of Pt #1 (right) and with antibodies which recognize the tranfected protein (left). In all cases, Pt #1 serum recognizes the transfected proteins. Note, however, in the m-amphiphysin 2 transfected cells, that Pt #1 serum does not recognize the linear elements representing m-amphiphysin 2 around membrane tubules (see insets representing higher magnification of regions outlined in rectangles). This observation suggests that only a subset of accessible m-amphiphysin 2 epitopes are labelled by the serum. Control = CHO cells transfected with mAmph2 and stained with secondary antibody only.

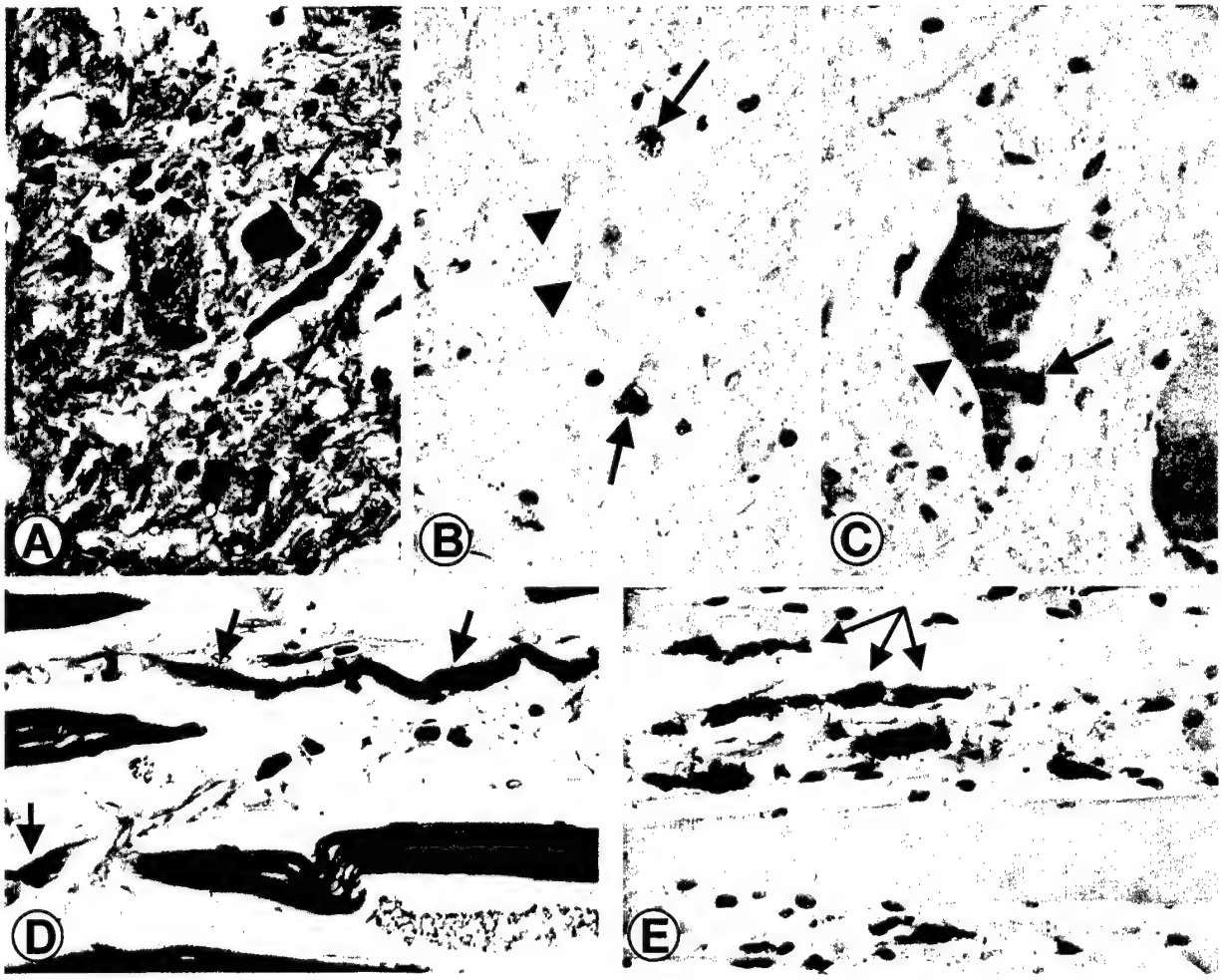

A
B
C
D
E

Fig. 2

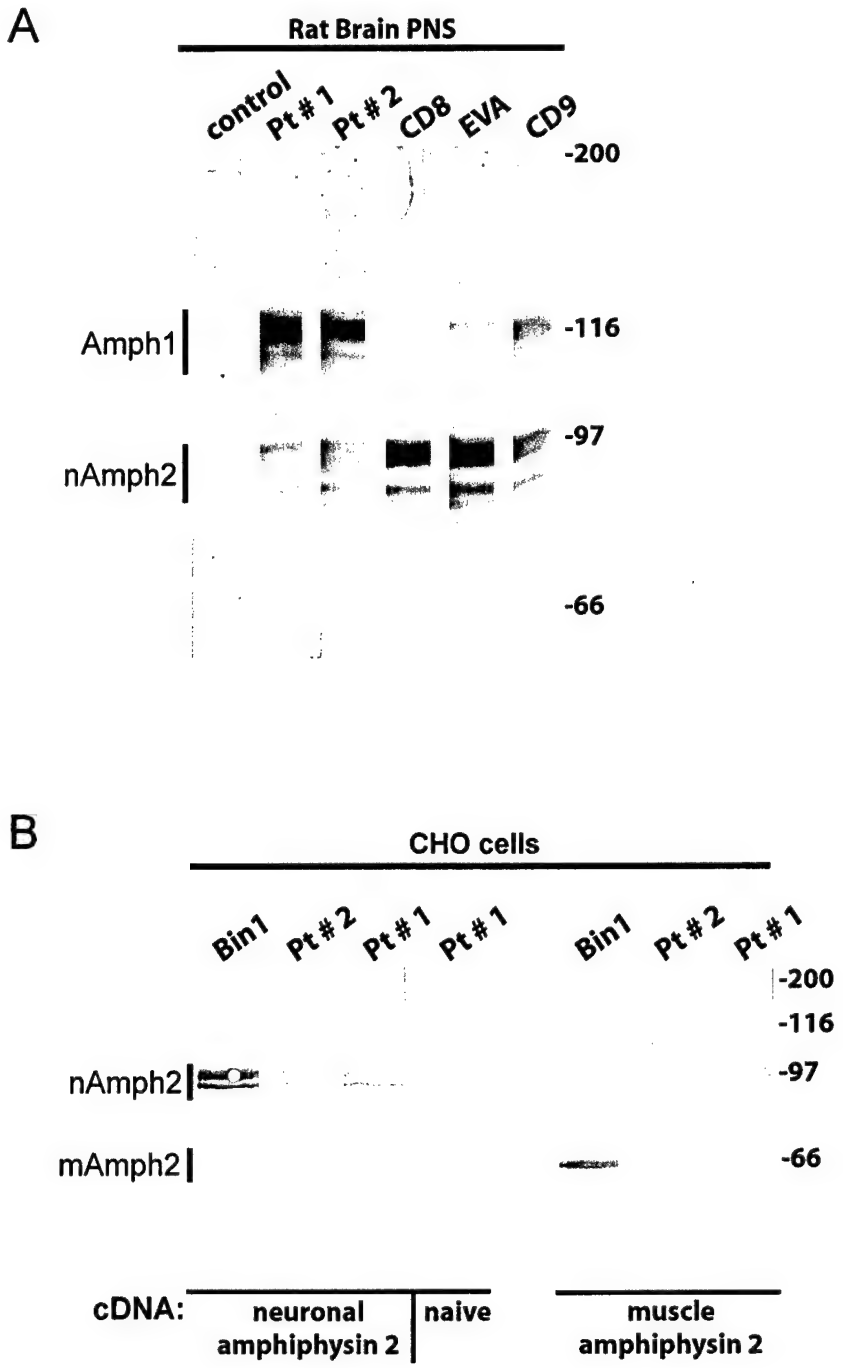
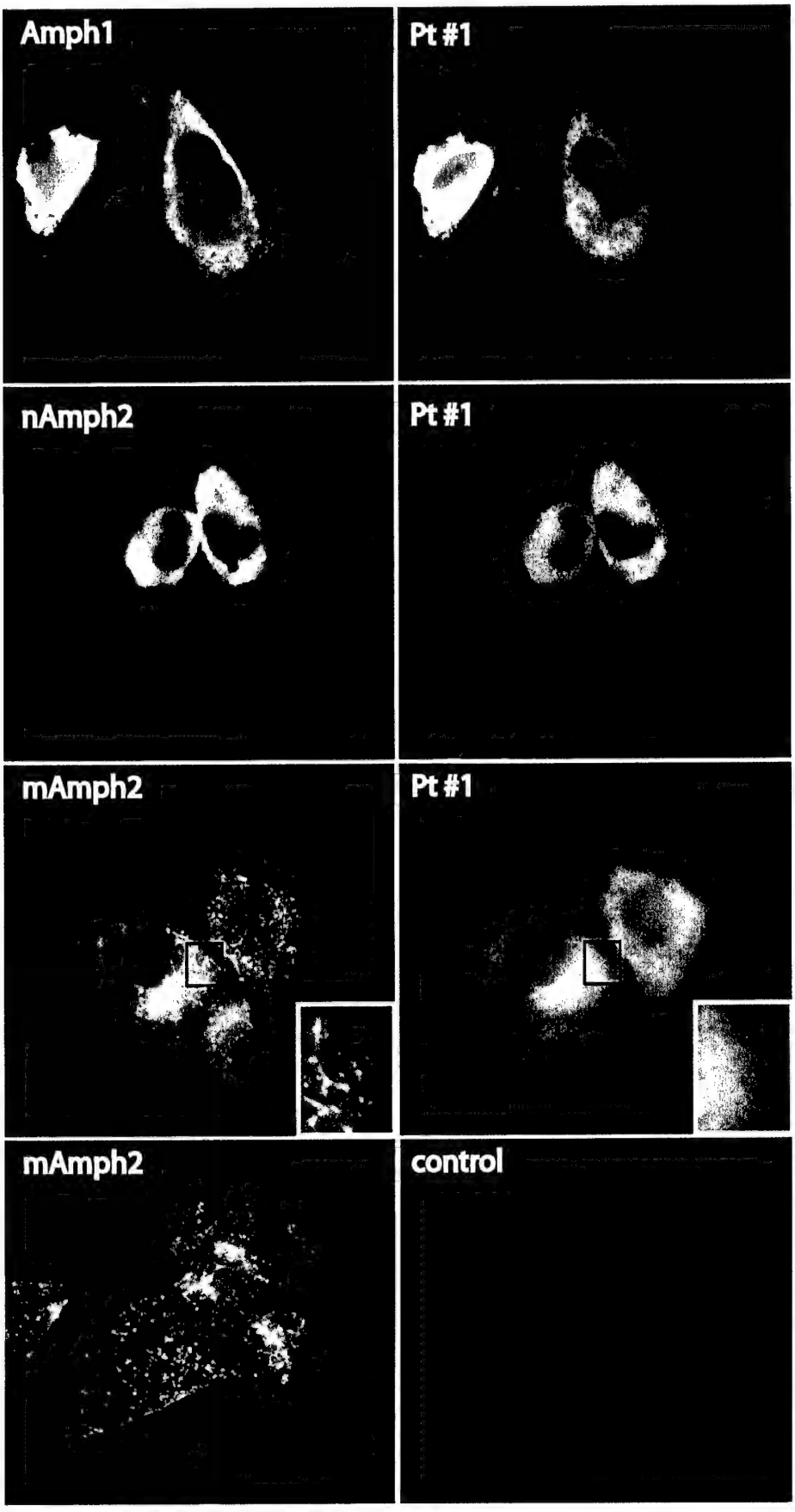

A
Rat Brain PNS
control
Pt # 1
Pt # 2
CD8
EVA
CD9
-200
Amph1
-116
nAmph2
-97
-66
B
CHO cells
Bin1
Pt # 2
Pt # 1
Pt # 1
Bin1
Pt # 2
Pt # 1
-200
-116
nAmph2
-97
mAmph2
-66
cDNA:
neuronal amphiphysin 2
naive
muscle amphiphysin 2

Fig. 3



Appendix 2

Quivering phenotype and altered distribution of K^+-channels in βIVspectrin Σ1 KO mice.

Sandra Lacas-Gervais[1]*, Jun Guo[2]*, Klaus-Peter Knoch[1], Matthew Rasband[4], Pietro De Camilli[2] and Michele Solimena[1,3].
[1]Experimental Center, Medical School Carl-Gustav Carus, Technical University, Dresden, Germany ; [2] Dept Cell Biology · Howard Hughes Medical Institute, Yale University, New Haven, CT, USA [3]Max-Planck-Institute for Molecular Cell Biology and Genetic, Dresden, Germany ; [4]University of Connecticut Health Center, Farmington, USA.

Being required for the proper clustering of ion channels at axon initial segments and nodes of Ranvier of myelinated neurons, βIV spectrin plays an important role in nerve conduction. βIV spectrin has been identified as the main target of autoimmunity in a patient with paraneoplastic lower motor neuron syndrome, which is caused by the destruction of myelinated motoneurons (Berghs *et al*, PNAS, 2001) The βIV spectrin gene undergoes extensive alternative splicing, leading to the expression of at least six isoforms, named Σ1 to Σ6. In the quivering mouse spontaneous mutations of several βIV spectrin isoforms cause auditory and motor neuropathies (Parkinson *et al*, Nature Genetics, 2001). To begin addressing the individual role of βIV spectrin isoforms, we generated isoform- specific antibodies and a knock out mouse in which only βIVΣ1 spectrin (250 kD), but not the smaller isoforms (160 kD and 140 kD), is absent. Lack of βIVΣ1 spectrin is sufficient to induce a quivering phenotype. Immunocytochemistry on brain sections shows an enrichemennt for βIV spectrins at the nuclear envelope of neurons, in addition to the previously reported concentration at axon initial segments and nodes of Ranvier. Staining of the nuclear envelope is not affected in βIVΣ1 -/- mice. On the contrary, immunolabeling is significantly reduced at axon initial segments, while the distribution of K^+ channel in the perinodal region is enlarged. Based on these data we conclude that βIVΣ1 spectrin is the specific isoform localized at axon initial segments and it is required for the restricted distribution of K+ channels.

* equal contributors

Abstract to the ELSO Meeting, September 2003, France

Appendix 3

DELETION OF THE SPECIFIC AND PH DOMAINS OF BETA IV SPECTRIN RESULTS IN DISRUPTENODES OF RANVIER.

Y Yang1, S Lacas-Gervais2, K Morest1, M Solimena2, MN Rasband1

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The node of Ranvier is a highly polarized structure characterized by the clustering of ion channels, cell adhesion molecules, and adaptor proteins. A recently identified cytoskeletal component beta IV spectrin, was reported at nodes of Ranvier and has been proposed to be involved in targeting and/or stabilizing nodal proteins. Alternative splicing of beta IV spectrin generates at least six isoforms. The longest of these, sigma 1, consists of an N-terminal actin-binding Calponin-homology (CH) domain, followed by 17 spectrin repeats, a 'specific domain' characterized by four EROES repeats, and finally a C-terminal pleckstrin homology (PH) domain. Of the remaining isoforms, only sigma 3 and 6 are predicted to contain the PH domain. We have examined mice (quivering3J or QV3J) with a mutation in the 'specific domain', resulting in truncation and loss of the PH domain. Homozygous mutant QV3J mice show ataxia that progressively worsens, followed by death at just a few months of age. At many nodes, nodal, paranodal, and juxtaparanodal proteins are delocalized and nodes appear to degenerate. In particular, immunostaining with sodium channel antibodies showed that in the CNS nodal clusters were twice the length of control littermates. Finally, while ultrastructural analysis of 3-4 month old mutant optic nerves showed that transverse bands were present, and myelin appeared to be appropriately compacted, nodes were twice the length found in control mice, with an average length of 1.85 micrometers. **Supported by NIH RO1NS44916, the Wadsworth Foundation and the A. von Humboldt Foundation.**

Abstract to the Society for Neuroscience Meeting, November 2003

Appendix 4

Principal Investigator/Program Director *(Last, first, middle):* De Camilli, Pietro

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Pietro De Camilli, M.D.	**Professor & Investigator**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Lyceum Manzoni, Milan, Italy	Maturita Classica	1996	Liceo Classico
University of Milano School of Medicine, Italy	M.D.	1972	
University of Pavia School of Medicine, italy	Post Grad.	1975	Medical Endocrinology

Positions & Honors

1972-1978 Assistant Professor, Department of Medical Pharmacology and CNR Center of Cytopharmacology, University of Milano, Italy

1978-1979 Postdoctoral Fellow, Department of Pharmacology (laboratory of Paul Greengard), Yale University School of Medicine

1979-1981 Assistant Professor, Section of Cell Biology, Yale University School of Medicine

1981-1987 Associate Professor, Department of Medical Pharmacology and CNR Center of Cytopharmacology, University of Milano, Italy

1987-1988 Visiting Associate Professor, Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York

1988-1992 Associate Professor with tenure, Department of Cell Biology, Yale University

1997-2000 Chairman, Department of Cell Biology

1992-present Professor, Department of Cell Biology; Investigator, Howard Hughes Medical Institute, Yale University School of Medicine

1978 - 1981: Fulbright -Hays Visiting Scholar in Pharmacology (Yale University); 1978: NATO fellowship (declined); 1978: Fellowship from the Muscular Dystrophy Association of America; 1979: Fellowship from the Muscular Distrophy Association of America; Since 1987: EMBO (European Molecular Biology Organization) member; 1989: Klingenstein Neuroscience Award; 1990: Max-Planck Research Prize (shared with Dr. R. Jahn, Münich); 1991: McKnight Research Project Award; 1995: DATTA Lecturer and Medal Recipient, Federation of European Biochemical Societies (Basel, Switzerland); 1997: Kroc Lecture on Diabetes, University of Washington; 1997: Member of the National Advisory Committee of the Pew Scholars Program for Biochemical Science; Member of the Italian Telethon Scientific Committee; 1997: Keith Porter Lecture, American Society for Cell Biology Meeting (Washington, DC); 2001: Member, American Academy of Arts and Sciences; 2001: Member, American National Academy of Sciences; 2002: Eighth Annual Kenneth F. Naidorf Memorial Lecture, Columbia University (New York, New York); 2002: Alex Novikoff Lecture, 2002 Gordon Conference on Lysosomes (Andover, New Hampshire)

Publications (Selected Recent Publications)

Rosin L, **De Camilli P**, Butler MH, Solimena M, Schmitt HP, Norgenthaler N, and Meinck HM. 1998. Stiff-man syndrome in a woman with breast cancer: an uncommon central nervous system paraneoplastic syndrome. *Neurology.* 50: 94-98.

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Appendix 5

BIOGRAPHICAL SKETCH

Michele Solimena, M.D., Ph.D.	POSITION TITLE Professor of Experimental Diabetology, TU-Dresden

A Education

1979	Lyceum Berchet, Milano, Italy	Degree: "Maturita" (Humanities)
1986	University of Milano School of Medicine, Italy	Degree: M.D. (Medicine)
1993	University of Milano School of Medicine, Italy	Degree: Ph.D. (Pharmacology)

B. Professional Experience

1986-1988:	Postdoctoral Fellow, Department of Pharmacology, Univ. of Milano, Milano, Italy
1988-1993:	Postdoctoral Fellow, Dept. of Cell Biology, Yale School of Medicine
1994-1999:	Asst. Prof., Dept. of Internal Medicine, Yale School of Medicine
1999-9/2001:	Assoc. Prof., Depts. of Internal Medicine and Cell Biology, Yale School of Medicine
10/2001-12/2002:	Group Leader, Max Planck Institute for Molecular cell Biology and Genetics, Dresden, Germany
01/2003-present	Professor for Experimental Diabetology, Faculty of Medicine, University of Technology Dresden

Honor and Awards

1987: Trabucchi Fellowship; 1996-1998; 1988: Levi Fellowship, Lincei's National Academy; 1988: Upjohn Prize; 1989: MDA Fellowship; 1990-1991: MDA Sydney-Blackmer Fellowship; 1992-1993: JDF Fellowship; 1994-1996: JDF Career Development Award; 1996-1998: ADA Research Award; 1996-1999: Donaghue Foundation New Investigator Award; 1997: NIH Director's Shannon Award; 1999-2002: ADA Research Award; 2001-2003: Wolfgang Paul Award, Alexander von Humboldt Foundation, Germany.

C. Selected publications

Solimena M, Folli F, Denis-Donini S, Comi G, Pozza G, De Camilli P, Vicari A. 1988. Autoantibodies directed against glutamic acid decarboxylase (GAD) in the cerebrospinal fluid and serum of a patient with Stiff-Man syndrome, epilepsy and type I diabetes mellitus. **New Engl. J. Med.** 318: 1012-20.

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Floyd S, Butler MH, Cremona O, David C, Freyberg Z, Zhang A, **Solimena M**, Tsutsui K, De Camilli P. 1998. Enhanced expression of amphiphysin in breast cancer. **Mol. Medicine** 4: 29-39.

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